

Small-Molecule Inhibition of UBE2T/FANCL-Mediated Ubiquitylation in the Fanconi Anemia Pathway

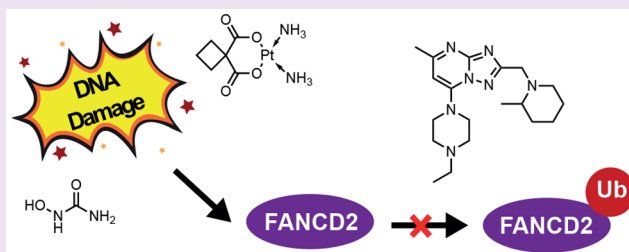
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S Supporting Information

ABSTRACT: The Fanconi anemia pathway orchestrates the repair of DNA interstrand cross-links and stalled replication forks. A key step in this pathway is UBE2T and FANCL-dependent monoubiquitylation of the FANCD2-FANCI complex. The Fanconi anemia pathway represents an attractive therapeutic target, because activation of this pathway has been linked to chemotherapy resistance in several cancers. However, to date, very few selective inhibitors of ubiquitin conjugation pathways are known. By using a high-throughput screen-compatible assay, we have identified a small-molecule inhibitor of UBE2T/FANCL-mediated FANCD2 monoubiquitylation that sensitizes cells to the DNA cross-linking agent, carboplatin.



Ubiquitylation is a post-translational modification that regulates a myriad of cellular processes. Proteins are ubiquitylated via an enzymatic cascade composed of E1 activating, E2 conjugating, and E3 ligating enzymes.¹ Because of the crucial physiological role of the ubiquitin system, its dysregulation is implicated in a growing number of human pathologies, including several cancers, developmental defects, immunodeficiencies, and neurodegenerative disorders.² Ubiquitylation is known to play key roles in a vast array of proteolytic and nonproteolytic regulatory mechanisms. One area in particular where ubiquitylation events are highly prevalent is in the DNA damage response (DDR).³

Genome integrity is continuously under attack from a barrage of exogenous and endogenous genotoxic agents such as ionizing radiation, ultraviolet light (UV) radiation and oxidative stress, and by errors in DNA replication itself. Fortunately, cells possess highly efficacious mechanisms—collectively known as the DDR—which are able to, among other things, detect DNA lesions, activate cell cycle checkpoints, and repair the damaged DNA.⁴

The Fanconi anemia (FA) pathway, also known as the FA/BRCA pathway, is required for the repair of DNA interstrand cross-links (ICLs).⁵ ICLs are among the most cytotoxic forms of DNA lesion, and occur when bases from opposite DNA strands become covalently attached to each other. ICLs inhibit essential processes such as replication and transcription and must be repaired or bypassed for the cell to survive.

ICL-inducing anticancer agents, such as platinum-based compounds (including cisplatin and carboplatin) and mitomycin C, have long been used in the clinic to treat a

range of malignancies including testicular, ovarian, head and neck, colorectal, bladder, and lung cancers.⁶ Although these chemotherapies are generally initially effective at cytoreduction, tumor recurrence and drug resistance commonly arise.⁷ Activation or upregulation of the FA pathway has been linked to chemotherapy resistance in several cancers; therefore, its inhibition is hypothesized to restore sensitivity to ICL-inducing agents.⁸

Currently, 22 genes are annotated as FA genes (FANCA to FANCW; <http://www2.rockefeller.edu/fanconi/mutate/>), with inactivation of any of these genes causing the genetic cancer predisposition syndrome termed Fanconi anemia.⁹ Key components of the FA pathway are the ubiquitin E2 enzyme, UBE2T (also known as FANCT) and the RING-type ubiquitin E3 ligase, FANCL.¹⁰ In response to the stalling of replication forks at sites of DNA ICLs, UBE2T functions with FANCL and the multiprotein FA complex to monoubiquitylate both subunits of the heterodimeric FANCD2-FANCI (ID) complex. The monoubiquitylated ID complex is then recruited to and retained at sites of ICL lesions and provides a platform for coordinating DNA repair events. When the repair process is completed, the ID complex is deubiquitylated and dissociated from the repaired ICL site by the USP1-UAF1 complex and released from the DNA.¹¹

Ubiquitin conjugation is dependent on many protein–protein interactions (PPIs), and the efficient formation and

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disassociation of protein complexes. Therefore, despite ubiquitin conjugating proteins possessing enzymatic activity, it is perhaps more apt to classify them as PPI targets. In drug and chemical probe discovery, such targets are viewed as challenging. This is perhaps reflected by the scarcity of selective small molecule inhibitors of ubiquitin conjugation pathways reported to date.¹²

To identify small-molecule inhibitors of the FA pathway, we developed a high-throughput screen (HTS) compatible assay based on the FA ubiquitylation cascade (see Figure 1a, as well

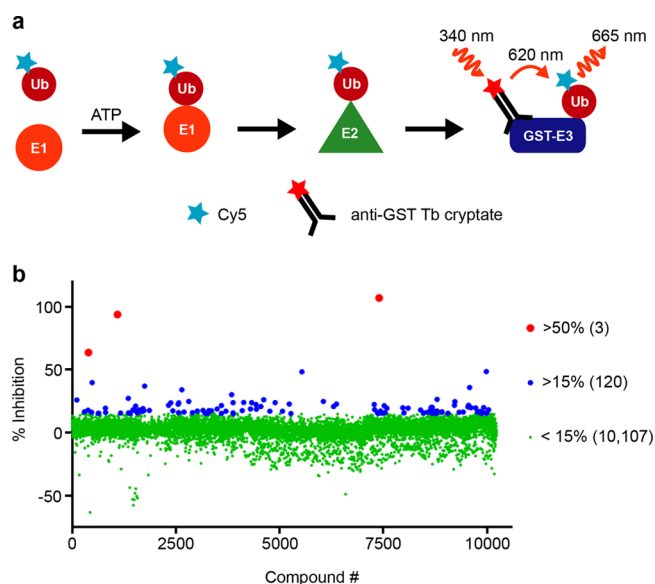


Figure 1. Screening for inhibitors of the FA pathway. (a) Schematic of the HTRF ubiquitylation assay. Ubiquitylation of GST-tagged E3 (FANCL_{RING}) by the E2 (UBE2T) places Cy5-labeled ubiquitin in close proximity to the anti-GST Tb cryptate. Excitation of the Tb cryptate donor results in FRET to the Cy5 acceptor. Simultaneous monitoring of the donor emission (620 nm) and acceptor emission (665 nm) allows for determination of the 665/620 ratio. (b) HTRF screen results showing average inhibition ($n = 2$) produced by compounds at 20 μ M (in-house diversity library; 10 111 compounds) and 10 μ M (Selleckchem epigenetic library; 119 compounds). Numbers given in parentheses represent the number of compounds per inhibition threshold.

as Figure S1 in the Supporting Information). Given the complexity of the full FA ubiquitylation cascade, we constructed a simplified ubiquitylation reaction that would be robust for HTS purposes yet still provide many relevant protein species for small molecules to interact with. The recombinant protein assay developed used homogeneous time-resolved fluorescence (HTRF) and contained Cy5-labeled ubiquitin, the E1 enzyme UBE1, the E2 enzyme UBE2T, and the RING domain (residues 275–375) of the E3 FANCL (FANCL_{RING}). FANCL_{RING} was used as a surrogate substrate for ubiquitylation in the absence of the FA core and FANCD2/FANCI complexes.

Subsequent screening of a leadlike diversity chemical library consisting of $\sim 10\,000$ compounds ($n = 2$) (robust Z score of >0.75) (Figure S2 in the Supporting Information) at a concentration of 20 μ M led to the identification of 120 primary hits, a hit rate of 1.17% (Figure 1b). The primary hit significance threshold (15% inhibition) was defined as three standard deviations above the mean inhibition across the entire screen. The compound library screened was specifically

curated to avoid compounds with known promiscuous mechanisms of action, such as redox cycling and cysteine reactivity.

Of the primary hits, the 32 most potent compounds—in relation to the observed percentage inhibition at 20 μ M—were selected for a 10-point dose–response study. Of the 32 compounds tested, 12 exhibited an IC_{50} value of $<100\ \mu$ M. Fresh stocks of these compounds were sourced from commercial vendors and IC_{50} values of $<100\ \mu$ M were obtained for 10 compounds (Figure S3; see the example for CU2 in Figure S4 in the Supporting Information). For most compounds, there was a good correlation between the data from the primary screen and the resupplied compounds (Table S1 in the Supporting Information).

The 10 compounds confirmed by dose–response were then tested in an orthogonal assay to rule out interference with the HTRF assay format. Thus, a modified ubiquitylation reaction was run with biotinylated ubiquitin and in the absence of the anti-GST-Tb antibody. The reaction components were then separated by SDS-polyacrylamide gel electrophoresis and immunoblotted. Notably, no ubiquitylation of FANCL_{RING} was observed in the presence of compounds CU1, CU2, CU9, CU10, or CU12 at 100 μ M, as indicated by the absence of the band corresponding to monoubiquitylated (mUb)-GST-FANCL_{RING} (Figure 2a, top panel). However, CU10 and CU12 also prevented the ubiquitylation of UBE2T (Figure S5 in the Supporting Information) and, in addition, somewhat impaired the autoubiquitylation of UBE1 (Figure 2a, bottom panel). Encouragingly, CU1, CU2, and CU9 did not affect ubiquitylation of either UBE2T or UBE1 (see Figure 2a, as well as Figure S5), thereby suggesting some target selectivity.

In order to explore the selectivity of CU1, CU2, CU9, CU10, and CU12, we conducted a counter-screen against other E2 and E3 combinations. Since members of the UBE2D family of E2s are known to ubiquitylate multiple substrates *in vitro*,¹³ a ubiquitylation assay featuring UBE2D1 and GST-FANCL_{RING} was performed. With UBE2D1, multiple ubiquitylated GST-FANCL_{RING} species could be observed (Figure 2b). As with UBE2T and GST-FANCL_{RING}, CU1, CU2, CU9, CU10, and CU12 essentially abolished the ubiquitylation of GST-FANCL_{RING}. CU10 and CU12 also prevented the formation of any ubiquitylated UBE2D1 species.

A further ubiquitylation assay was performed with an alternative E3, RNF8, which also plays important roles in the DDR.¹⁴ In contrast to GST-FANCL_{RING}, long polyubiquitin chains were attached to GST-RNF8 by UBE2D1 (Figure 2c). Consistent with our previous observations, CU10 and CU12 appeared to inhibit the ubiquitylation of all species in the assay. In support of our data indicating the selectivity of CU1, CU2, and CU9, these compounds had no detectable effects on ubiquitylation of GST-RNF8 and UBE2D1.

The lack of an inhibitory effect on the ubiquitylation of UBE1, UBE2T, UBE2D1, and RNF8 by CU1, CU2, and CU9 suggested that these compounds bind to a specific species in the ubiquitylation reactions and are not acting via a pan-assay interference (PAIN)-type mechanism. Furthermore, CU1, CU2, and CU9 appeared to exhibit some degree of selectivity for FANCL-dependent ubiquitylation, although not necessarily selectivity for a particular E2. We did not observe binding of CU2 to UBE2T by differential scanning fluorimetry, ligand-observed NMR, or native mass spectrometry (unpublished data). Taken together, these findings suggest that the target for CU1, CU2, and CU9 is potentially the RING domain of

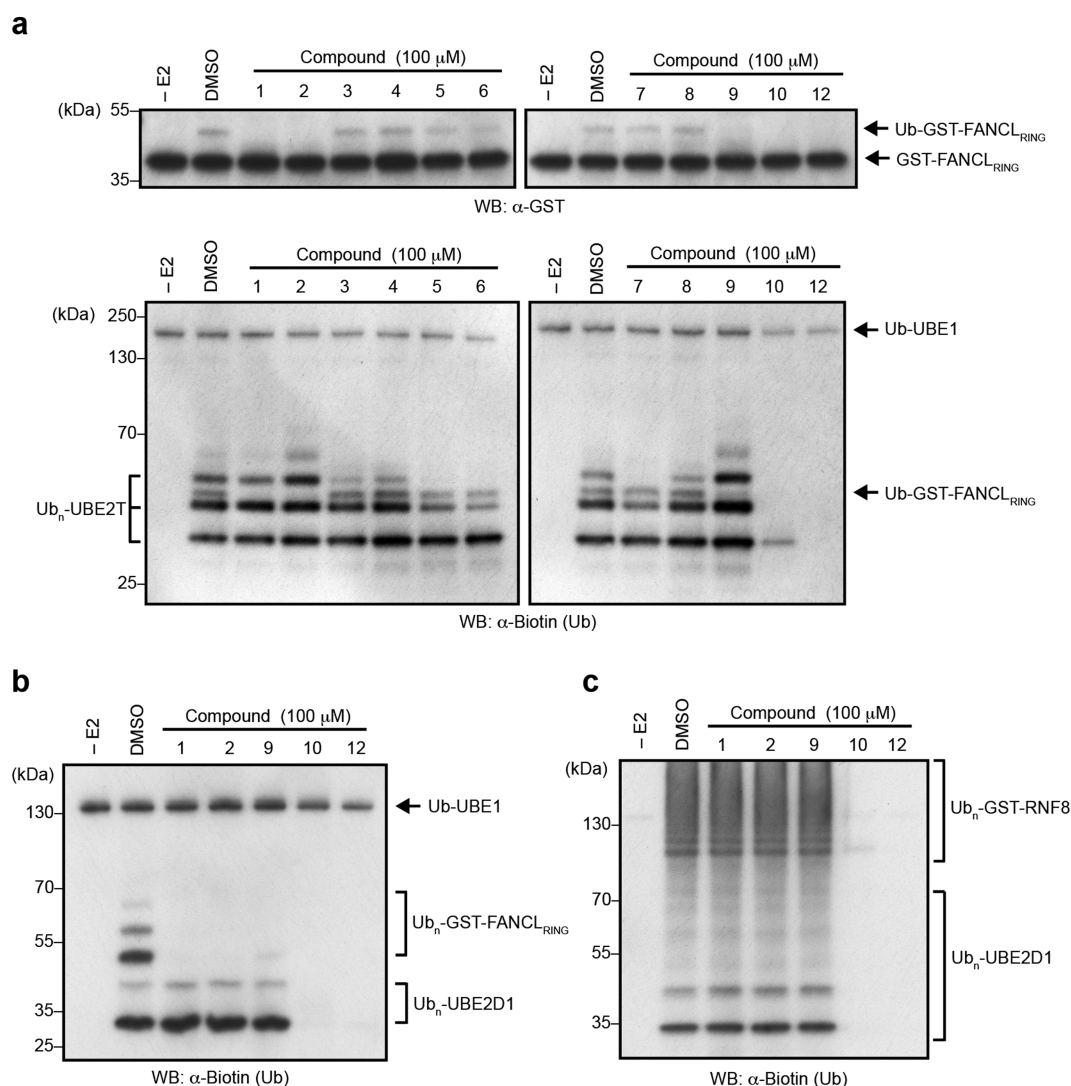


Figure 2. Gel-based ubiquitylation assays with confirmed HTRF hits. **CU1**, **CU2**, and **CU9** inhibit the ubiquitylation of GST-FANCL_{RING}, but not GST-RNF8. Western blots (WB) probed for GST or biotin as labeled. Reactions contained 20 nM His₆-UBE1, 50 nM E2, 250 nM GST-E3, 500 nM biotinylated ubiquitin: (a) E2 = UBE2T, E3 = GST-FANCL_{RING}, 30 min of reaction at 37 °C; (b) E2 = UBE2D1, E3 = GST-FANCL_{RING}, 30 min of reaction at 37 °C; and (c) E2 = UBE2D1, E3 = GST-RNF8, 10 min of reaction at 37 °C.

FANCL or a protein complex comprised thereof; however, further experimental evidence will be required to confirm this.

Next, **CU1**, **CU2**, and **CU9** were evaluated for their ability to inhibit FANCL-dependent ubiquitylation events in cells. **CU10** and **CU12** were deprioritized for cellular evaluation due to their lack of selectivity in the *in vitro* gel-based ubiquitylation assays. The only known *in vivo* function of UBE2T and FANCL is their direct role in the monoubiquitylation of the heterodimeric FANCD2-FANCI complex, which occurs in response to certain types of DNA damage. Hydroxyurea (HU) is a potent inhibitor of the enzyme ribonucleotide reductase (RNR) and is an antineoplastic drug used in the clinic.¹⁵ Inhibition of RNR limits *de novo* dNTP synthesis, causing depletion of cellular dNTP pools, which, in turn, leads to stalled and collapsed replication forks and cell cycle arrest in S-phase. Because of the role of the FA pathway in the stabilization and repair of stalled and collapsed replication forks, HU is a strong inducer of the UBE2T and FANCL-dependent monoubiquitylation of FANCD2 (mUb-FANCD2).¹⁶

U2OS cells were treated with HU and the formation of mUb-FANCD2 in the presence of **CU1**, **CU2**, and **CU9** was evaluated relative to a DMSO control. Following a 1-h preincubation with the compounds, cells were treated with 2.5 mM of HU for 24 h. Biochemically, at 100 μM, **CU1**, **CU2**, and **CU9** completely inhibited the ubiquitylation of GST-FANCL_{RING}. However, consistent with the decrease in potency often observed in cellular assays, relative to *in vitro* biochemical assays,¹⁷ when cells were treated with **CU1**, **CU2**, and **CU9** at 100 μM, no significant decrease in the level of mUb-FANCD2 was observed (see Figure S6 in the Supporting Information). Accordingly, cells were treated with a higher concentration of compounds in further experiments. In these studies, we also decreased the assay time from 24 h to 8 h to mitigate potential compound instability. Thus, we found that cells tolerated **CU2** and **CU9** at 500 μM, while **CU1** was found to be cytotoxic above 300 μM. Notably, both **CU1** (200 μM) and **CU2** (500 μM) reduced the level of mUb-FANCD2 in response to HU treatment (see Figures 3a and 3b). **CU2** (500 μM) also reduced the level of mUb-FANCD2 generated in response to cisplatin (10 μM) treatment for 6, 12, and 24 h (Figure 3c).

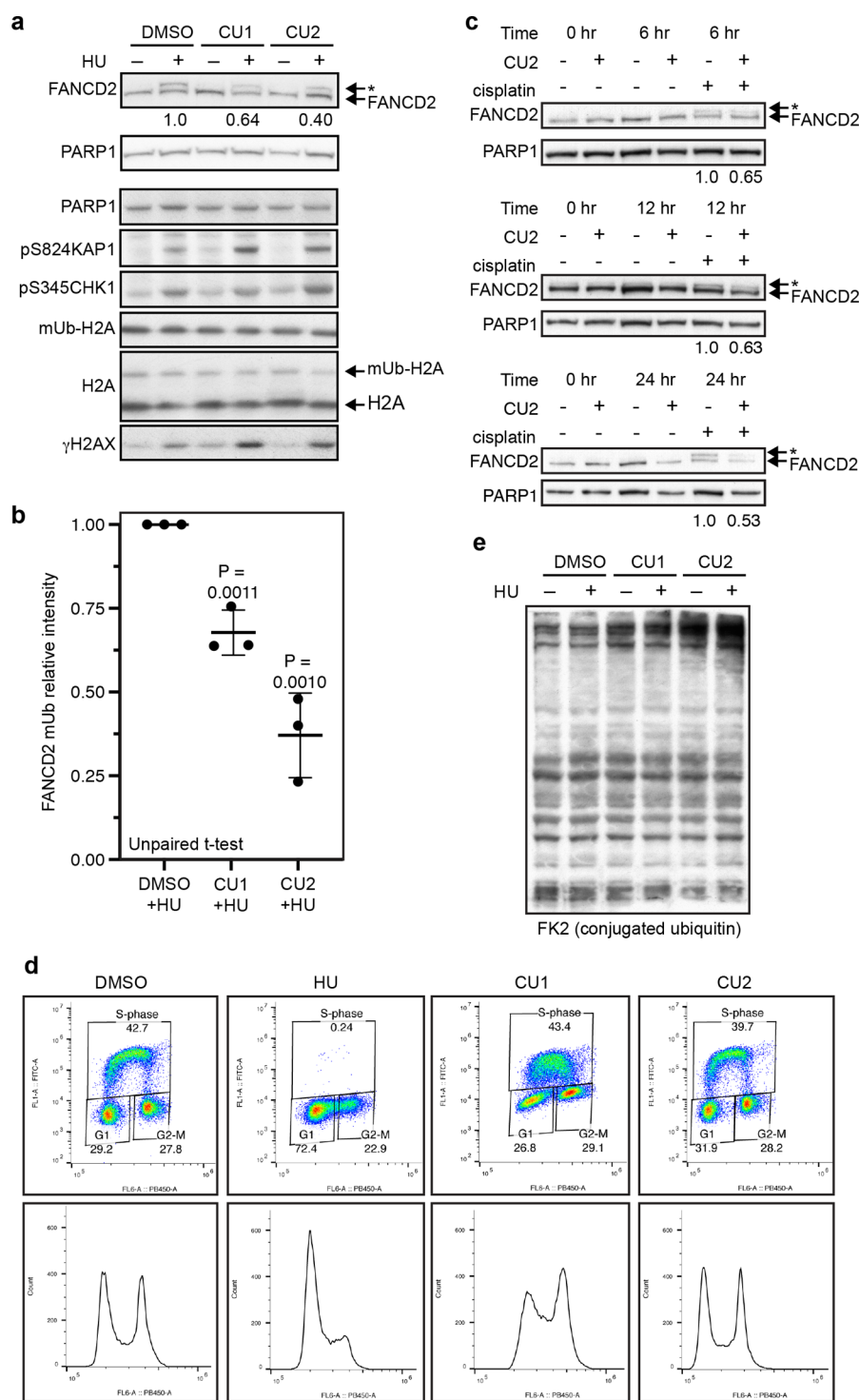


Figure 3. Cell-based assays with CU1 and CU2: (a) Western blot showing that the formation of mUb-FANCD2(*), in response to HU treatment (2.5 mM, 8 h), is impaired in U2OS cells in the presence of CU1 (200 μ M) or CU2 (500 μ M). Ratio = mUb-FANCD2/FANCD2 (normalized to DMSO control). mUb-FANCD2 was resolved on a separate gel. PARP1 was used as a loading control for each gel, as well as to assess apoptosis. (b) Quantification of the reduction in mUb-FANCD2, in response to HU treatment (2.5 mM, 8 h) in the presence of CU1 (200 μ M) or CU2 (500 μ M). Mean calculated from three independent experiments. (c) Western blot showing that formation of mUb-FANCD2 (denoted by an asterisk symbol, *) in response to cisplatin treatment (10 μ M) for 6, 12, and 24 h is impaired in U2OS cells in the presence of CU2 (500 μ M). Ratio = mUb-FANCD2/FANCD2 (normalized to DMSO control). (d) Cell cycle analyses based upon EdU incorporation in cells treated with HU (2.5 mM, 8 h) in the presence or absence of CU1 (200 μ M) or CU2 (500 μ M). HU treatment was used for gating purposes. (e) Western blot showing that CU1 (200 μ M) or CU2 (500 μ M) do not affect total protein ubiquitylation with or without HU treatment (2.5 mM, 8 h), as assessed by probing with the FK2 antibody.

As DNA damage induced by HU is cell-cycle-dependent, it was necessary to establish whether CU1 and CU2 might

simply prevent cells from entering the S-phase, which would also manifest itself as a reduction in mUb-FANCD2. This was

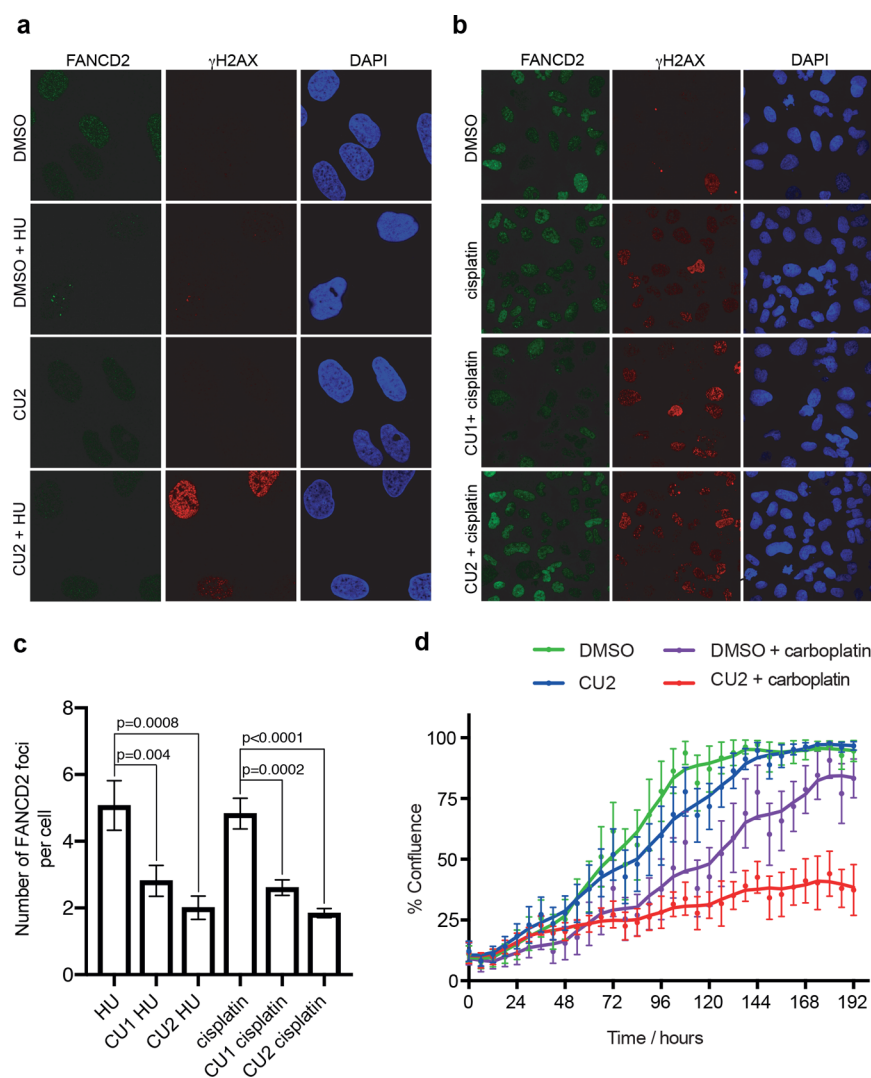


Figure 4. Further cell-based assays with CU1 and CU2: (a, b) Representative immunofluorescence images of U2OS cells treated with HU (2.5 mM, 8 h) or cisplatin (10 μ M, 8 h) in the presence or absence of CU1 (200 μ M) or CU2 (500 μ M). (c) Quantification of the number of FANCD2 foci in cells treated as in panels (a) and (b). Data accumulated over three independent experiments, each consisting of three technical replicates. (d) IncuCyte analysis of U2OS cells treated with or without carboplatin (15 μ M) and CU2 (250 μ M). Mean calculated from three wells, four images per well. (HU = DMSO + HU, cisplatin = DMSO + cisplatin).

determined by analysis of several biomarkers linked to DNA damage in cycling cells. Activated CHK1 (pS345 CHK1) phosphorylates CDC25A, inducing the transient arrest of cells in the S-phase, or in G2 before the onset of mitosis.¹⁸ Comparable levels of pS345 CHK1 were observed in cells treated with either CU1 or CU2, relative to the DMSO control, indicating that the compounds were not preventing cells from entering the S-phase (Figure 3a). These observations were also confirmed by cell cycle analysis. However, a slightly lower EdU (5-ethynyl-2'-deoxyuridine) incorporation intensity, but not S-phase index, was observed following CU1 treatment, suggesting that it may slow progression through the S-phase (Figure 3d).

Following the generation of DNA DSBs, ATM, ATR, and/or DNA-PKcs mediated phosphorylation of histone H2AX generates the phosphorylated species, γ H2AX.¹⁹ In accord with our conclusion that CU1 and CU2 did not markedly affect progression of cells into and through the S-phase, when used to treat cells in combination with HU, CU1 or CU2 did not decrease and, in fact, somewhat increased the levels of

γ H2AX, relative to cells treated with HU plus DMSO control (Figure 3a). Furthermore, these increased levels of γ H2AX suggested that larger numbers of DSBs were generated in HU-treated cells incubated with CU1 or CU2, compared with cells treated with HU and DMSO. Following HU treatment, DSBs are formed if stalled replication forks are not stabilized and subsequently collapse. Therefore, we concluded that CU1 and CU2 appeared to act by impairing the ability of cells to stabilize and repair stalled replication forks. This is consistent with CU1 and CU2 inhibiting the formation of mUb-FANCD2, which would otherwise play a role in stabilizing stalled replication forks, thus preventing their collapse and the formation of DSBs.

In response to DSB formation, the transcriptional repressor and RING finger protein, KAP1, is released from chromatin, following its ATM-mediated phosphorylation on Ser824.²⁰ In agreement with our findings with γ H2AX, increased levels of pS824 KAP1 were observed with both CU1 and CU2, which provides further evidence of the existence of more DSBs/collapsed replication forks (Figure 3a). An increase in the

number of DSBs could also have been caused by apoptosis. In cells undergoing apoptosis, PARP1 is cleaved from a full-length 116 kDa protein into 89 and 24 kDa polypeptides by caspase-3.²¹ These PARP1 cleavage fragments were not observed when cells were treated with HU plus CU1 or CU2, indicating that the effects we observed on H2AX and KAP1 phosphorylation were not due to apoptosis and, instead, were likely due to the compounds inducing a defect in the FA pathway (Figure 3a).

To investigate if the compounds were affecting more widespread ubiquitylation events in the cell, the monoubiquitylation status of histone H2A was assessed (mUb-H2A; Figure 3a). The polycomb repressive complex 1 (PRC1) mediates ubiquitylation of histone H2A through its E3 ligase subunits, RING1A and BMI1, in combination with UBE2D3.²² In addition, the total level of monoubiquitylated and polyubiquitylated conjugates in the cell was assessed by immunoblotting of cell extracts with the FK2 antibody, which recognizes conjugated ubiquitin (Figure 3e). mUb-H2A and total protein ubiquitylation levels were not detectably affected in the presence of CU1 or CU2, suggesting that these compounds are not pan-ubiquitylation inhibitors.

In response to DNA damage, mUb-FANCD2 accumulates at sites of DNA lesions to form foci, promote DNA-damage sensing, activate cell-cycle checkpoints, and promote DNA repair.²³ Therefore, we employed high-throughput high-content indirect immunofluorescence studies to examine the effect of CU1 and CU2 on mUb-FANCD2 focus formation. The experimental format used was similar to the immunoblot-based experiments, excepting that, following treatment with HU (2.5 mM for 8 h) or cisplatin (10 μ M for 8 h), cells were fixed, permeabilized and stained with specific FANCD2 primary and fluorescent secondary antibodies. In addition to monitoring mUb-FANCD2 foci, γ H2AX was evaluated as a marker of DNA damage induction, and cell nuclei were visualized by 4',6-diamidino-2-phenylindole (DAPI) staining. We observed a clear reduction in the number of FANCD2 foci per cell in CU1- or CU2-treated cells in the presence of HU or cisplatin, relative to HU or cisplatin alone (Figures 4a, 4b, and 4c). These observations are in accord with the immunoblot-based experiments wherein reductions in mUb-FANCD2 were observed (Figure 3a). Although γ H2AX levels appear to be higher in the HU-treated cells (Figure 3a), as was the case in the immunoblot assay, the *p*-value of this increase did not pass the statistical threshold (*p* < 0.05). No such apparent increase was observed in the case of cisplatin treatment (see Figure S7 in the Supporting Information). The quantitative results—namely, no reduction in γ H2AX positive cells treated with CU1 or CU2 in combination with HU or cisplatin—are consistent with the cell cycle analyses indicating that CU1 or CU2 lead to no reduction in S-phase indices.

Finally, to investigate if CU1 and CU2 could sensitize cells to a more clinically relevant chemotherapeutic agent, quantitative live cell analyses were performed using the IncuCyte platform. As the FA pathway is involved in the repair of ICLs, cells were treated with the ICL-inducing chemotherapeutic drug, carboplatin. For this experiment, U2OS cells were seeded at a relatively low (<10%) density and preincubated with CU1 or CU2 for 1 h. Carboplatin was then added to the cells and images were taken every 6 h for 8 days in order to monitor growth and proliferation of the cells. Although 500 μ M CU2 was used in the immunoblot- and immunofluorescence-based experiments, this concentration was found to be cytotoxic/cytostatic upon longer cellular

exposures. In contrast, CU2 at 250 μ M had a minimal effect on cell growth, with cells reaching 100% confluency after 6 days, a similar situation to when the cell line was treated with DMSO alone (Figure 4d). The addition of 15 μ M carboplatin to DMSO-treated cells led to a clear reduction in proliferation rate, and, after 8 days, the cells had still not reached 100% confluency. The combination of 250 μ M CU2 with 15 μ M carboplatin led to an even more pronounced decrease in cell proliferation/growth, with the cells being <50% confluent after 8 days (Figure 4d; as shown in Figure S8 in the Supporting Information, while the effect of CU1 was also examined, it was found to strongly inhibit cell growth at concentrations of >50 μ M and the combined effect of 50 μ M CU1 with 15 μ M carboplatin was far less pronounced than that observed for CU2).

In summary, the small-molecule screening campaign and follow-up studies described herein represent a potential starting point for the development of a chemical probe or therapeutic agent targeting the FA pathway. One of the compounds identified, CU2, exhibits promising selectivity in biochemical ubiquitylation assays and also demonstrated activity against the FA pathway in cells. Further work will be necessary to elucidate the precise nature of the protein or protein complex to which CU2 binds. Given the relatively low molecular weight (357 Da) of CU2, there is certainly scope to improve its cellular potency and target selectivity through chemical synthesis. CU1 represents a less-attractive starting point, in view of the cytotoxicity that we observed. A more potent inhibitor of the FA pathway could find clinical application in sensitizing cancer cells to DNA cross-linking agents (such as carboplatin or mitomycin C). A selective FA pathway inhibitor could also be utilized in the identification and exploitation of synthetic lethal interactions in cancer cells with certain genetic backgrounds.²⁴

MATERIALS AND METHODS

Detailed methods are described in the Supporting Information.

ASSOCIATED CONTENT

Supporting Information

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Experimental procedures, Figures S1–S9, and Tables S1–S4 (PDF)

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Notes

The authors declare no competing financial interest.

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